Tumor Promoters and Cocarcinogens in Tobacco Carcinogenesis

by D. Hoffmann,* S. S. Hecht,* and E. L. Wynder*

Cigarette smoke induces carcinoma of the larynx in Syrian golden hamsters and is active as a tumor promoter in hamsters pretreated with a low dose of a PAH, nitrosamine, or nitrosamide. These tumorigenic effects are only observed with total smoke, but not with the gas phase alone. This demonstrates that the tumorigenic agents reside primarily in the particulate phase. According to fractionation experiments, a number of four- and five-ring aromatic hydrocarbons serve as the major tumor initiators in tobacco smoke. Tumor promoters reside primarily in weakly polaric neutral subfractions and in the weakly acidic portion of the particulate matter and include certain unsaturated hydrocarbons and phenolic compounds. Cocarcinogenic activity is a characteristic feature of tobacco smoke and its particulates. Among the cocarcinogens formed during combustion are catechols and certain nontumorigenic aromatic hydrocarbons and terpenes. Nicotine may also serve as a cocarcinogen as is indicated by preliminary data. The action of tumor promoters and cocarcinogens in tobacco carcinogenesis, the precursors for tobacco smoke promoters and cocarcinogens, and methods for their reduction in smoke are discussed.

Introduction

Epidemiological studies have established a causal relationship of cigarette smoking with cancer of the larynx and of the lung as well as an association of cigarette, cigar and pipe smoking with cancer of the oral cavity, pharynx, larynx and esophagus. It has also been shown that cigarette smokers have an increased risk for cancer of the pancreas, kidney and urinary bladder (1). Tobacco chewing and snuff dipping have been associated with cancer of the mouth (1, 2). The exposure to sidestream smoke as an indoor air pollutant has recently been incriminated as a possible risk factor for lung cancer among nonsmokers (3-5).

All of these observations tend to support the concept that tobacco smoke is a complete carcinogen, a fact, also borne out by smoke inhalation studies and by tumor induction with tobacco "tar" in the skin of mice and rabbits and in the subcutaneous tissues and trachea of rats (6-8).

In the human setting, there is actually only one suggestion that tobacco smoking may be a tumor promoter and/or cocarcinogen and this relates to the role of tobacco smoking in uranium miners. Occupational exposure to α-particles from radon and

radon-daughters represents the causative factor for increased lung cancer risk among uranium miners (1, 9, 10). This is documented by lung cancer incidence rates of 71 per 100,000 nonsmoking uranium miners in the U.S. per year. Lung cancer incidence rates per year for individuals who are not subjected to the occupational uranium exposure are 11 per 100,000 for nonsmokers and 44 per 100,000 for heavy cigarette smokers. This contrasts sharply with the rate of 422 for lung cancer among cigarette-smoking uranium miners (11) (Fig. 1).

The initiator-promoter concept which this observation in man suggests, regards the relatively low exposure to α-radiation as the initiator and the long term exposure to cigarette smoke as the tumor-promoter phase in a two-stage carcinogenesis model. In fact, studies in experimental tobacco carcinogenesis confirm that the tumor formation occurs as a multistep process wherein different tobacco and smoke constituents play different roles.

It is the purpose of this review to discuss laboratory studies which have sought to define these tobacco carcinogens as well as their respective roles in the formation of tumors.

Inhalation Experiments

Successful assays for tumorigenic activity of inhaled tobacco smoke were developed only after

*Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY 10595.
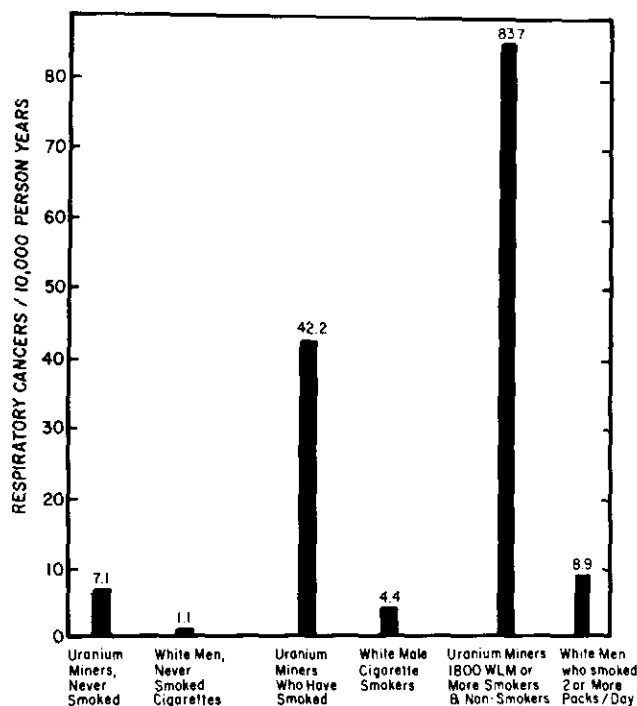
FIGURE 1. Respiratory cancer rates among uranium miners by cigarette usage and radiation exposure compared with rates among nonminers. Data of Archer et al. (11).
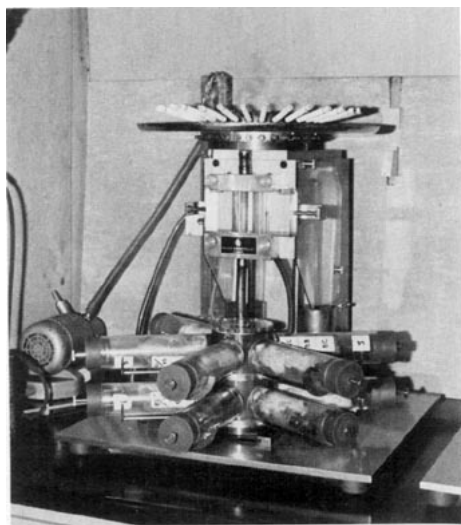

FIGURE 2. The Hamburg II smoke inhalation device for hamsters.

1. NASAL APEX
2. NASAL FUNDUS
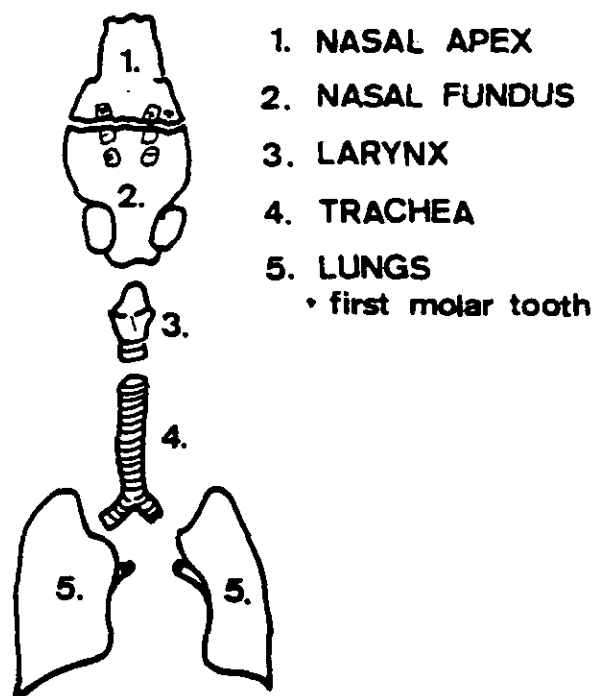3. LARYNX
4. TRACHEA
5. LUNGS
 * first molar tooth

FIGURE 3. Dissection pattern of the respiratory tract for determining localized smoke particulate exposure of hamsters by measuring radioactive deposites from inhaled (^{14}C) dotriacontane-labeled cigarette smoke. From Mohr and Reznik (7).

many years of searching for a suitable model. Toxicity of cigarette smoke and the anatomical nasal features of rodents who were the most accessible and affordable test objects, presented barriers to the successful induction of tumors in the respiratory system. However, the Syrian golden hamster has finally emerged as the most suitable model (2, 3), although Nettesheim et al. have recently also succeeded with rats (4). The hamsters are exposed to diluted cigarette smoke (1:15) in tubular compartments of a smoking device shown in Figure 2 (15). Each exposure is 10 minutes once, twice or three times daily, five times per week, for the duration of the hamsters' lifetime. In these groups of 80 hamsters each (7), 11.3, 30 and 30.6% of the animals developed pre- invasive carcinoma of the upper larynx (Fig. 3) Larynx tumors were not observed in the control groups, nor in hamsters exposed only to the gaseous phase of tobacco smoke. Trachea and bronchi of the animals were free of neoplasms.

Utilizing this experimental design, we attempted to document the initiator:promoter concept by sensitizing the hamsters with subthreshold doses of known carcinogens prior to long-term inhalation exposure to certain smoke constituents. Application of 7,12-dimethylbenz(a)anthracene (DMBA) to the larynx or intratracheal instillation of DMBA once weekly for 2 weeks was followed by exposure to air-diluted smoke (7:1) of nonfilter cigarettes (1972-1973), 5 days per week (16). Equal-sized groups received either DMBA or smoke exposure and a fourth group served as vehicle control, each hamster receiving 0.2 mL of a 0.5% saline solution twice in successive weeks and then being observed.
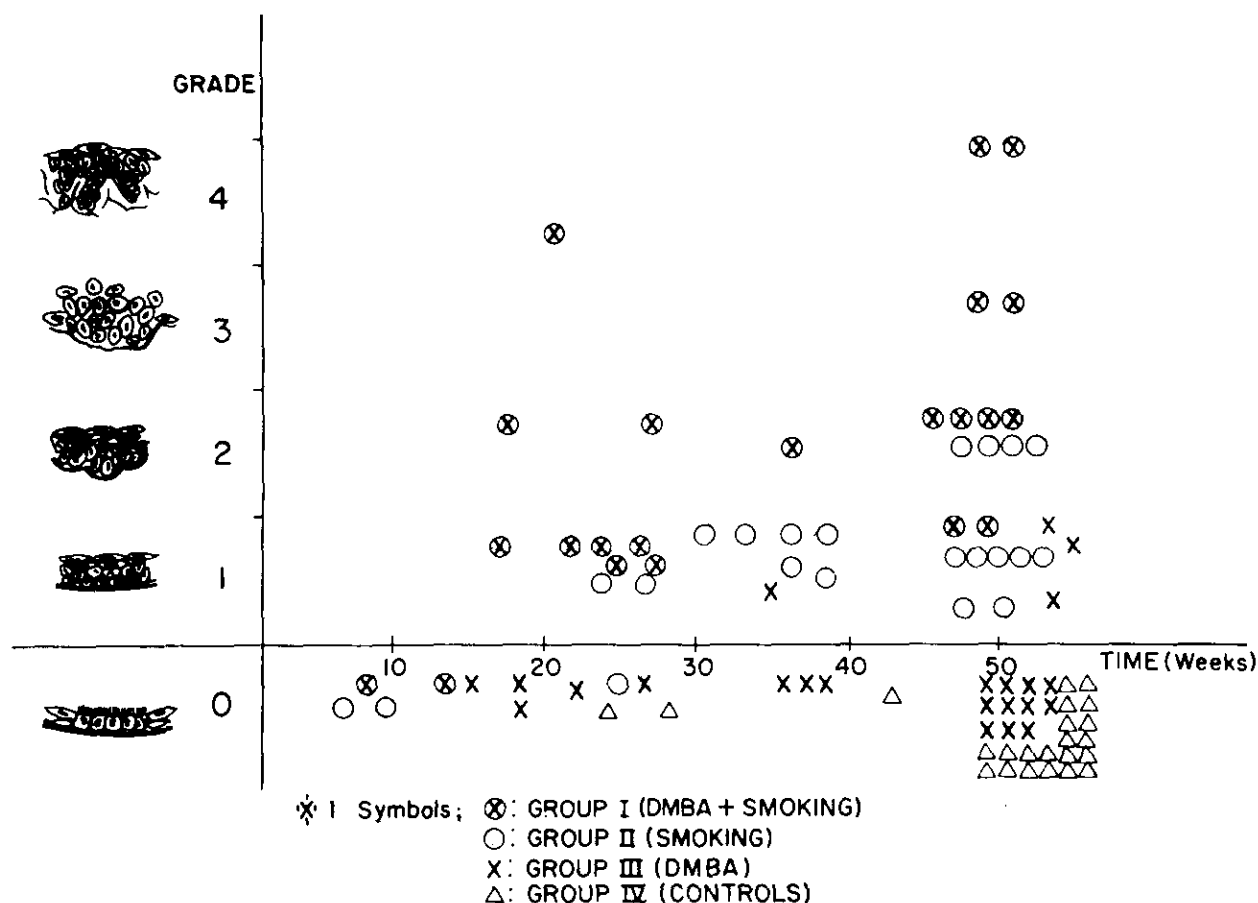
FIGURE 4. Epithelial changes in hamster larynx induced by DMBA and smoke exposure, smoke exposure alone, or DMBA alone. Three hamsters each in Groups I and II and two hamsters each in Groups III and IV were lost due to cannibalism. Data of Kobayashi et al. (16).

After 48 weeks of inhalation and another 4 weeks of observation, the animals were killed and necropsied. Hyperplasia and neoplastic lesions and tumors developed in the larynx of the hamsters receiving both DMBA and smoke in significantly higher incidence, and earlier than in the group treated by smoke exposure only. In addition, the DMBA-sensitized hamsters showed also squamous cell metaplasia of the nasal cavity and papilloma of the oral cavity as well as pharyngeal papillomas, thus indicating promoter activities for cigarette smoke (Fig. 4). Dontenwill et al. (17) had also measured the effects of treatment with DMBA in addition to smoke exposure and found that the numbers of tumors obtained in the oral cavity, pharynx, esophagus, forestomach and trachea were significantly increased over the tumor yield induced by smoke exposure alone.

With 4 mg diethylnitrosamine (DENA) as initiator we had exposed Syrian golden hamsters to the smoke of 10 cigarettes daily in a 72 liter inhalation chamber for 6 months (15). The same device was also used to measure the effects of the gaseous smoke phase and the effects of a vaporized aldehyde-acid mixture corresponding to ratios of smoke aldehydes and acids on DENA-induced respiratory neoplasms (Fig. 5). Although the set-up did not exclude artifacts during aging of the smoke, this experiment strengthened the hypothesis that total smoke as well as gaseous smoke constituents increase DENA-induced tumor formation in the hamster trachea (Table 1).

Nitrosamines as initiators were also used by Dontenwill et al. (17, 18), Wehner et al. (19) and by Karbe and Köster (20) in essentially similar smoke inhalation assays with the Syrian golden hamster.

Table 2 summarizes the principal alterations observed in major studies by hamster pretreatment with DMBA, diethylnitrosamine (DENA), N-methyl-N-nitroso-urea (NMU) and asbestos (15, 17-21). All of
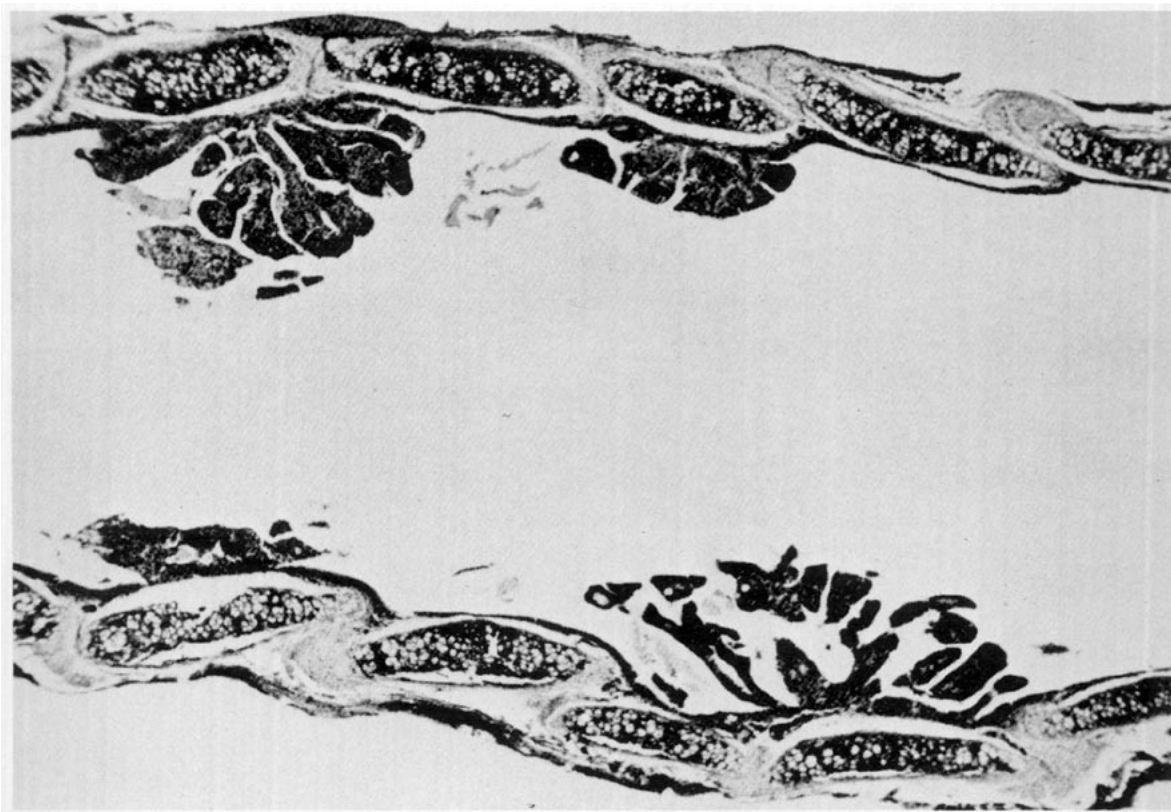
FIGURE 5. Multiple benign papillomas of the trachea of a Syrian golden hamster given 4 mg of DENA and exposed to an admixture of smoke acids and aldehydes for 6 months. HE; × 15.

Table 1. Passive inhalation of aged cigarette smoke by Syrian golden hamsters.[a]

		Exposure, months[b]				
		1	3	4	5	6
1	4 mg DENA +			1	2	6
	cigarette smoke	12	9	7	5	0
				1	4	17
2	4 mg DENA +					8
	aldehydes	12	12	12	12	0
						20
3	4 mg DENA + acids[c]					9
		12	12	12	12	0
						23
4	4 mg DENA +					11
	aldehydes + acids[c]	12	12	12	12	0
						34
5	4 mg DENA +					6
	methyl nitrite	12	12	12	12	0
						21
6	Methyl nitrite[c]	10	9	9	9	7[d]
						3
7	4 mg DENA	12	10	8	6	0
						4

[a] Data of Hoffmann et al. (15).

[b] Upper number in each column denotes number of hamsters with tracheal papillomas, number in center of column = surviving hamsters and lower number = total number of tracheal papillomas.

[c] In concentrations as in 15 min aged smoke of 10 cigarettes.

[d] Terminated after 10 months, no tumors were observed.

these studies except one with DENA by Wehner et al. (21) indicate the tumor promoter potential of cigarette smoke, whereby the severity of histopathologic grading of lesions appears to be related to the initiator potential of the pretreatment as well as to the dose and duration of tobacco smoke exposure. Studies by Karbe and Köster have shown that smoke exposure by inhalation increased the incidence of malignant tumors in NMU-initiated hamsters about 4-fold (20).

The observations that respiratory tumor development is largely dose-dependent on the promoter, in this case tobacco smoke, provide a plausible explanation for the fact that the risk of lung cancer in ex-smokers declines progressively when the promoter insult ceases (22, 23) as was discussed (24) by Wynder during this symposium (Fig. 6).

Tumor-promoting effects of tobacco smoke in hamsters who were pretreated with tobacco-specific N-nitrosamines (TSNA) have not been established. It would be of major importance to study such effects because TSNA are formed from tobacco alkaloids, occur in smoke at levels up to 8 μg per cigarette and are organ-specific carcinogens (25) (Table 3).

Table 2. Principle alterations in animals exposed to DMBA, DENA, and asbestos in addition to smoke exposure compared to their single-substance controls.

| Treatment	Principal alterations	Reference
DENA + smoke	Increase in incidence of papillomas in the trachea, bronchi, and lower region of the larynx	(17) (15)
NMU + smoke	Increase in epidermoid carcinoma (grade 5) in the larynx and increase in precancerous lesions (grade 4) in larynx and pharynx	(20)
DMBA + smoke	Increase in incidence of tumors in the oral cavity, pharynx, esophagus, stomach, trachea, liver and ovaries	(17) (16)
Asbestos + smoke	Development of laryngeal tumors. Absence of tumors in the bronchi, trachea and pharynx	(17) (19)
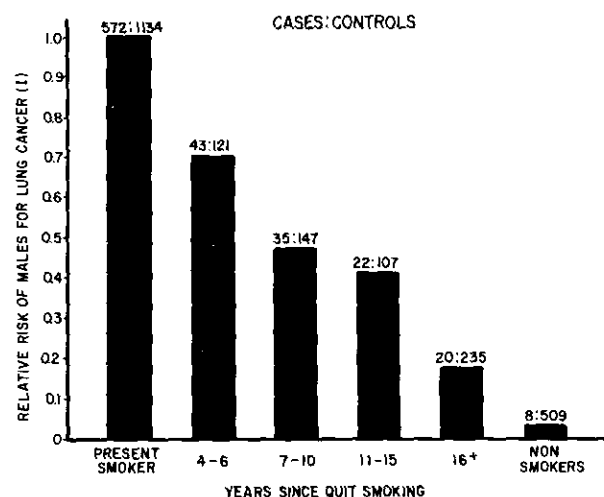
FIGURE 6. Decline of lung cancer in ex-smokers. Wynder and Stellman, (22).

Studies with the Particulate Phase

The gaseous phase of tobacco smoke does not by itself induce tumors of the respiratory tract in laboratory animals (8, 17). Thus, it may be deduced that the major carcinogenic activity resides in the particulate phase, more commonly known as "tar." Benign and malignant tumors have been induced with tobacco tar in the skin and ear

When efforts were made to pinpoint the carcinogens in the tar by fractionations, bioassays · on mouse skin revealed that the observed tumorigenic activities were composite effects of the intricate mixture of compounds which constitutes cigarette smoke particulates. Chemical identification of such compounds has led to the knowledge that certain polynuclear aromatic hydrocarbons are the major tumor initiators, but that the neutral and weakly acidic fractions of tar contain various types of cocarcinogens and tumor promoters (Fig. 7) (26, 27). It is also important that the smoke particulates as a whole have dose-related promoting effects on DMBA- initiated mouse skin (Fig. 8).

Neutral Portion

A few years ago, we reported that the phenanthrene subfraction and the polynuclear aromatic hydrocarbon (PAH) subfractions of the neutral portion of cigarette "tar" are active as cocarcinogens when applied to mouse skin, together with 0.003% BaP (26, 27, 36, 37). Three and four-ring PAH are known cocarcinogens rather than active tumor initiators. It became, therefore, an important task to determine them individually and to examine whether the neutral portion contained yet other cocarcinogens, since PAH are primarily pyrosynthesized during the incomplete combustion of tobacco by the same mechanism.

We approached the determination of cocarcinogenic agents by fractionation of cigarette "tar" with several distribution steps and column chromatography. This resulted in five neutral subfractions (Fig. 9). The two PAH- containing subfractions were

Table 3. Tobacco specific N-nitrosamines in tobacco products.[a]

Nitrosamines	Tobacco, ppm	Chewing tobacco or snuff, ppm	Cigarette smoke, μg/cigarette	Cigar smoke, μg/cigar
N'-Nitrosonornicotine	0.2-45	3.5 -77	0.2 -3.7	3.2-5.5
NNK[b]	0.1-35	0.8 - 4.7	0.12-0.44	1.9-4.2
N'-Nitrosoanabasine	0.0-0.01	0.04- 1.9	0.0 -0.15	n.d.[c]
N'-Nitrosoanatabine	0.6-13	0.8 -44	0.15-4.6	1.7-1.9

[a] Data of Hoffmann et al., (15).

[b] NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
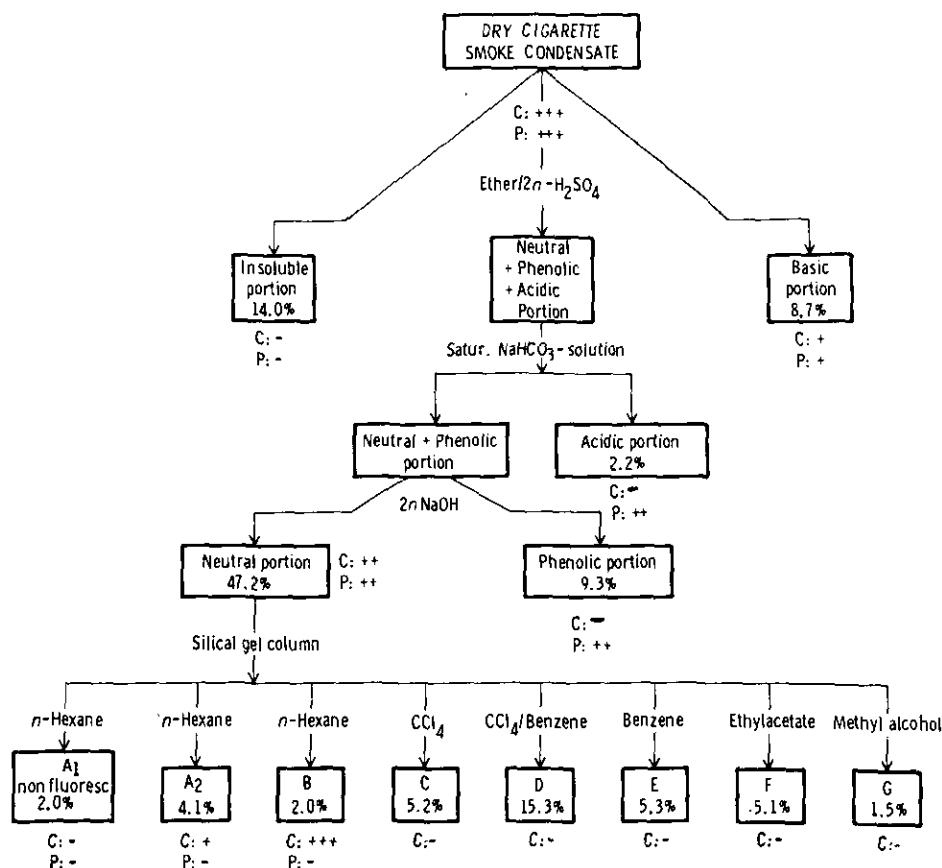
[c] Not determined.

FIGURE 7. Fractionation of tobacco smoke particulates and relative carcinogenic and promoter activities of major fractions. Data of Wynder and Hoffmann (8).
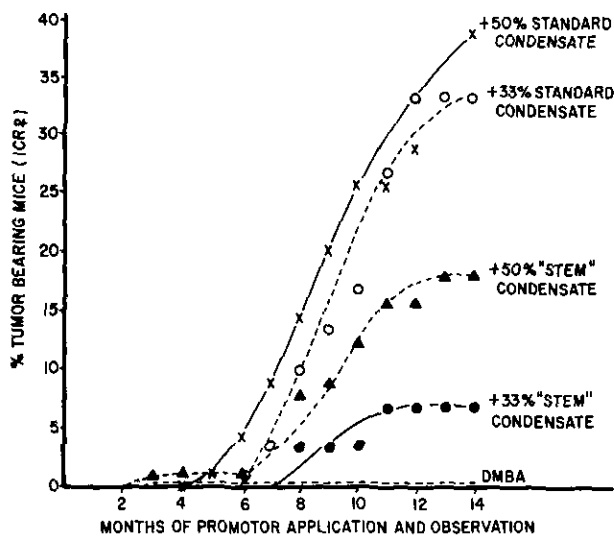

FIGURE 8. Tumor-promoting activities of cigarette smoke *condensate*. Initiator: 150 g dimethylbenz(a)anthracene (DMBA). Promoters: 50% solutions of smoke condensates of *standard cigarette or of cigarettes made of stems*; 90 mice tested per group. 33% solutions of both test materials were tested on 30 mice per group. Use of 150 g DMBA as initiator with acetone as promoter yielded no tumors in 50 mice. Data of Wynder and Hoffmann (37).

further separated into two portions at ~70°C: acetone-solubles (PAH) and the acetone-insolubles (free of PAH). All seven neutral subfractions were bioassayed on mouse skin for their cocarcinogenic activity together with 0.003% BaP. (The fractions were applied in proportions relative to their amounts in undiluted tar). None of the fractions were active as complete carcinogens in the chosen concentrations, however, the acetone-solubles and acetone-insolubles of the alkylnaphthalene and PAH subfractions were active as cocarcinogens (Fig. 10).

In-depth chemical analytical studies of the acetone-solubles of the alkylnaphthalene fraction revealed the presence of methyl- and dimethylnaphthalenes, methyl- and dimethylbiphenyls, methyl- and dimethylfluorenes, methylanthracenes, and methylphenanthrenes.

The insolubles of the PAH subfraction yielded as major compounds solanone, phytone, 2-hexadecanone, 2-heptadecanone, 3-heptadecanone, 2-octadecanone, 2-nonadecanone, 3-nonadecanone, 2-eicosanone and 3-docosanone. It is important that these long-chain methyl and ethyl ketones and/or their precursors are plant-specific and are found in these concentrations only in tobacco and the resulting
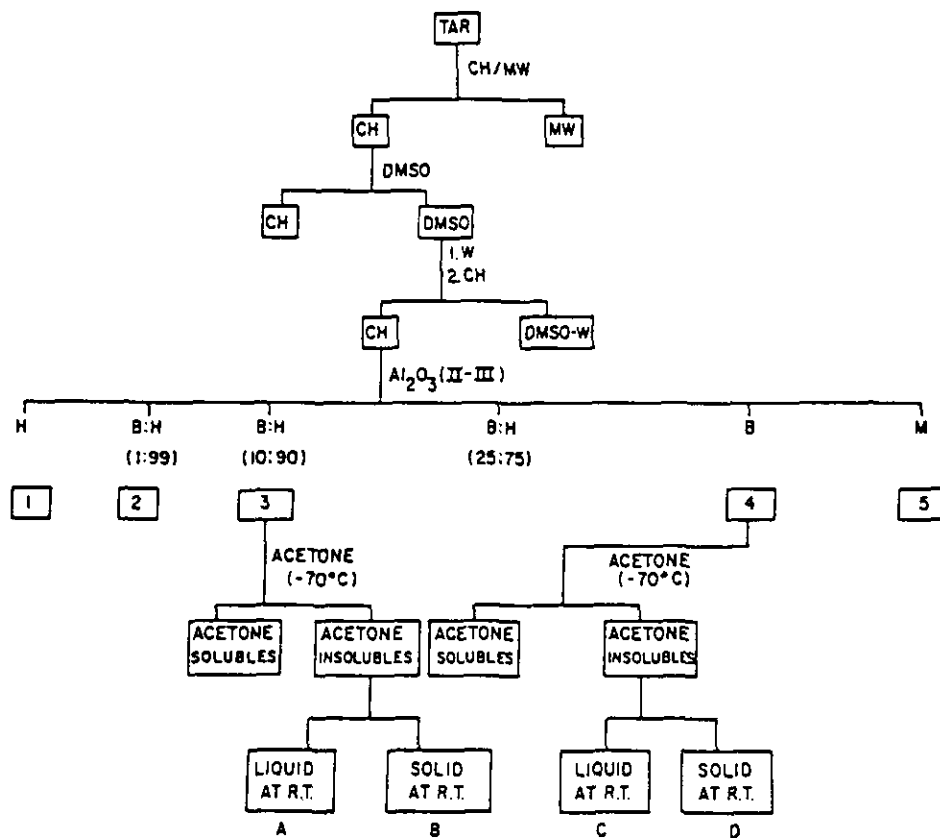
FIGURE 9. Fractionation of cigarette tar for bioassays to determine cocarcinogenic activity of neutral subfractions. Data of American Health Foundation.

smoke. They account for 0.5-1.5% of the cigarette smoke condensate. Their individual cocarcinogenic potential needs to be determined.

Weakly Acidic Portion

Constituents of the weakly acidic fraction of tobacco tar were shown to act as tumor promoters or as cocarcinogens respectively (8, 27-29, 37). Coapplication of HPLC subfractions of the weakly acidic portion of tar with 0.003% BaP on mouse skin identified catechol as a major component of materials with cocarcinogenic activity (27), thus confirming studies by Van Duuren et al. (28). However, other weakly acidic compounds, though of lesser activity, contribute to the cocarcinogenic potential of the weakly acidic portion (Fig. 11). The verification of dose responses for catechol with BaP and the testing of other constituents of the active subfractions are still in progress.

It is of particular interest that the cocarcinogenic activities of active subfractions in these tests was expressed in terms of increased percentage of tumor-bearing animals as well as in terms of total tumor yield (Table 4). This is thus a true additive effect rather than merely an acceleration of tumor development as was observed with certain neutral smoke constituents. Tumor promotion in the classical sense by weakly acidic smoke constituents was demonstrated particularly for phenol in our studies on mouse skin with BaP as initiator (30), although Van Duuren found partial inhibition of BaP activity by phenol (31).

In view of such contradictory evidence and because of the fact that tumor-promoting activity and cocarcinogenic activity are not necessarily always correlated, further studies on metabolic processes during initiation and promotion, respectively during cocarcinogen application need to be undertaken.

Nicotine as a Possible Cocarcinogen

Discussion of cocarcinogenic activities of tobacco smoke would hardly be complete without alluding to the role of nicotine, especially since Bock and collaborators found that tobacco extracts had tumor promoting activity on DMBA-initiated mouse skin. However, this activity requires the concurrent
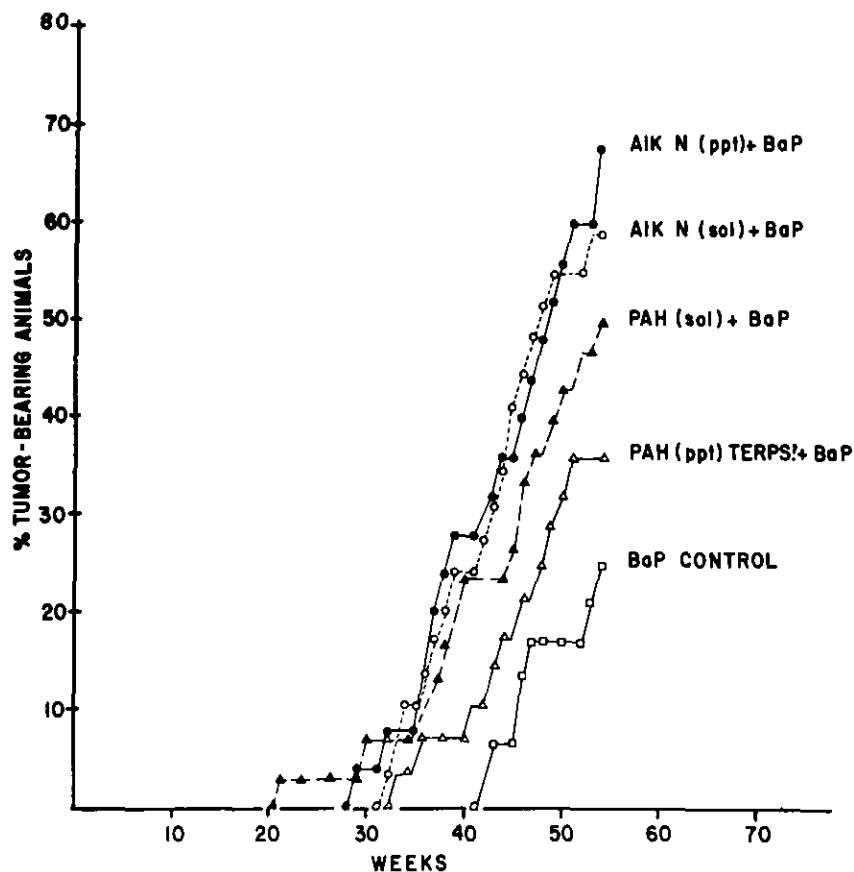
FIGURE 10. Bioassay data on mouse skin (ICR♀) from tests for cocarcinogenic activity of neutral subfractions of tobacco smoke condensate (30 mice per group). AIK N = neutral alkanes, PAH = polynuclear aromatic hydrocarbons, BaP = benzo(a)pyrene, ppt = precipitate, sol = solubles. Data of American Health Foundation.

presence of two agents, one of large molecular weight (LM), insoluble in organic solvents, and the other of small molecular weight (SM), soluble in organic solvents (32). It is suggested that the SM agent could be nicotine (33) while the LM fraction with the highest activity may consist of tobacco leaf pigments.

Model studies by Bock (34) on enhancement of BaP-TPA carcinogenesis by nicotine showed that such an effect was not due to the alkaloid's specific involvement in either initiation or promotion. The enhancement of carcinogenesis was also not a consequence of the metabolic conversion of nicotine to either cotinine or NNO (Table 5). Thus, Bock concluded "if metabolism of nicotine is critical for its activity, some other as yet unidentified metabolite must be involved."

Bock's studies on nicotine as a copromoter did point to a factor which has proven to be critical in a number of other investigations of cocarcinogenic effects, namely the relative ratio of the stimulant to the initiating carcinogen. In coapplication, variation of this relative ratio can produce effects ranging from enhancement to inhibition.

We have seen such effects in studies with benzo-(a)pyrene in coapplication with various molecular ratios of phenanthrene, pyrene and fluoranthene (35).

Furthermore, the concentration, sequence and frequency of application are critical in simple models but more so in complex admixtures (36, 37). Bock has shown that in an initiation-promotion protocol nicotine did not enhance the activity of TPA with an initiator dose of 125 μg DMBA.

Summary

The study of tumor promotion and cocarcinogenic effects in tobacco carcinogenesis has pointed to several classes of compounds which enhance the carcinogenic potential of tumor initiators that are germane to tobacco as well as to other known
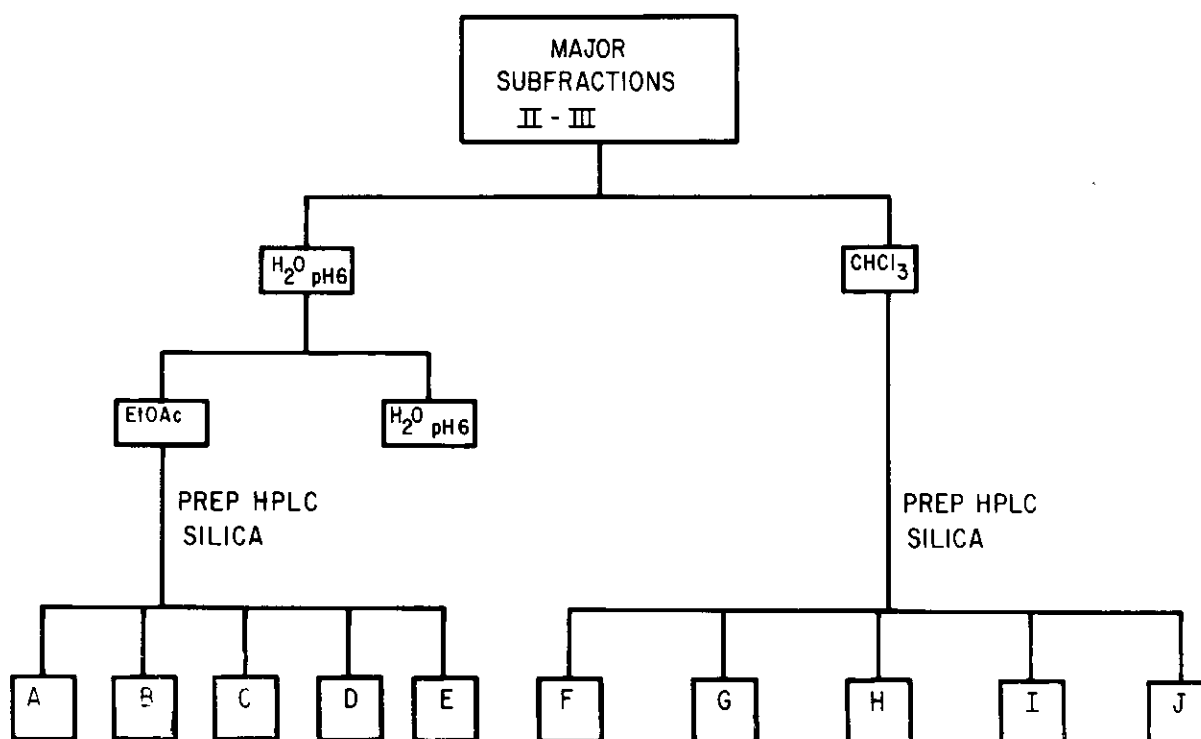
FIGURE 11.   Subfractionation of major subfractions II and III of the weakly acidic fraction of smoke condensates. Data of Hecht et al.
(29).

Table 4. Cocarcinogenicity of subfractions of the weakly acidic fraction[a,b]

BP and subfractions	Effective no. of animals	Mean latency period, wk	Animals surviving 52 wk, %	Animals with		Skin tumors/ mouse
				Skin tumors, %[c]	Squamous cell carcinoma (skin), %	
BP (0.003% in acetone)	28	41.0	90	14	11	0.1
BP (0.003% in ethanol)	30	39.5	87	3	3	0.2
BP (0.003%) + subfraction A (0.25%)	30	41.7	57	73*	64*	1.4
BP (0.003%) + subfraction B (0.16%)	30	48.2	80	60*	47*	0.9
BP (0.003%) + subfraction C (0.014%)	29	43.9	73	52*	38+	0.5
BP (0.003%) + subfraction D (0.014%)	30	43.4	83	33	27	0.3
BP (0.003%) + subfraction E (0.16%)	30	42.5	83	10	3	0.1
BP (0.003%) + subfraction F (1.2%)	29	43.3	47	76*	66*	2.1
BP (0.003%) + subfraction G (0.25%)	30	47.1	80	47*	33+	0.5
BP (0.003%) + subfraction H (0.36%)	30	43.6	67	73*	70*	1.4
BP (0.003%) + subfraction I (0.19%)	28	43.5	73	61*	50*	0.9
BP (0.003%) + subfraction J (0.51%)	30	46.0	83	23+	7	0.3

   [a] Data of Hecht, et al. (29).
   [b] Each group consisted of 30 Ha:ICR female Swiss mice. Solutions were applied five times weekly for 52 wk. Subfractions A-D and F-I were applied in acetone, Subfractions E and J were applied in ethanol.
   [c] One skin tumor was observed in the acetone control group, and no skin tumors were observed in the groups treated with ethanol or with each fraction without BP.
   * Significant, $p<0.01$.
   + Significant, $p<0.05$.

Table 5. Effect of nicotine metabolites on carcinogenic activity of B[a]P-TPA in acetone[a,b]

Test solution	Number of mice	Number of mice at risk[c]	Mice with skin tumors		Number of mice with skin cancer	Comparison with B[a]P-TPA alone	
			Number	%		χ^2	p
B[a]P-TPA alone[d]	75	75	37	49	22	—	—
B[a]P-TPA + 2.5 mg/mL nicotine	50	50	39	78	19	42	<0.001
B[a]P-TPA + 5 mg/mL nicotine	50	38	31	82	15	68	<0.001
B[a]P-TPA + 2.5 mg/mL cotinine	45	45	26	58	18	1.8	N.s.[e]
B[a]P-TPA + 5 mg/mL cotinine	45	45	29	64	18	4.6	0.03
B[a]P-TPA + 2.5 mg/mL NNO	45	45	10	22	8	13.8	<0.001
B[a]P-TPA + 5 mg/mL NNO	45	45	12	27	10	10.2	<0.01

[a] Data of Bock et al. (32).

[b] B(a)P = benzo(a)pyrene; TPA = tetradecanoylphorbol acetate.

[c] Number alive at 10 weeks when the first tumor appeared.

[d] 10 μg B[a]P plus 0.6 μg TPA per mL; 0.2 mL applied 10 times a week.

[e] Not significant.

tumor initiators. Among these are noncarcinogenic polycyclic aromatic hydrocarbons and their alkylated derivatives, acids and aldehydes of the gaseous phase of smoke, catechols and phenols and, possibly nicotine. These compounds enhance carcinogenic processes induced by polynuclear aromatic hydrocarbons, N-nitrosamines and likely other as yet unidentified tumor initiators.

Delineation of the mechanisms that lead to specific activities of the various classes of cocarcinogenic or tumor promoting compounds would indicate possibilities for chemopreventive approaches to tobacco carcinogenesis.

In practical terms, tobacco carcinogenesis continues to be most effectively reduced by diminishing the levels of initiators, promoters and cocarcinogens in smoke, since the carcinogenic as well as tumor promoting effects of the smoke particulates are clearly dose-related.

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